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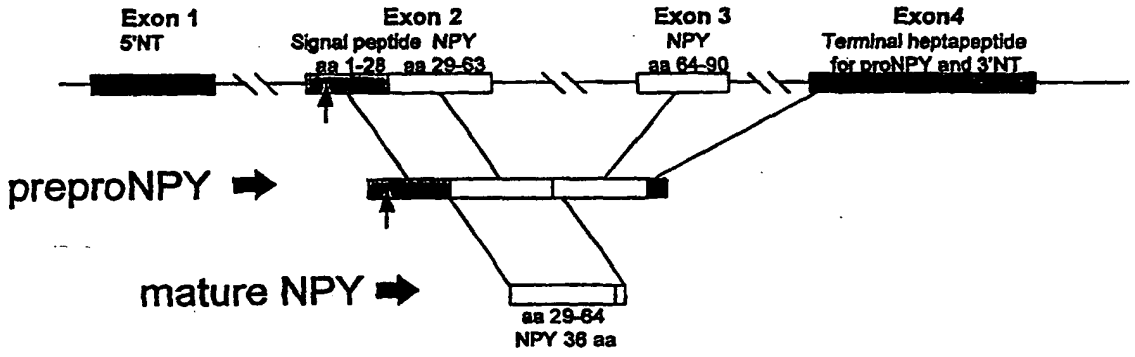

(57) Abstract

The invention relates to a DNA sequence comprising a nucleotide sequence encoding a prepro–neuropeptide Y (preproNPY) where the leucine amino acid in position 7 of the signal peptide part of said preproNPY has been replaced by proline. The invention concerns further the mutant signal peptide as such or associated with any other cleavage product of preproNPY, methods for the determination, in a biological sample, of said DNA sequence or said peptide. Furthermore, this invention relates to a method for diagnosing a predisposition for increased serum cholesterol or LDL cholesterol level in a human subject, and to methods for treating a human subject diagnosed for predisposition for increased serum cholesterol or LDL cholesterol. Transgenic animals carrying either the mutant sequence or the normal sequence are also within the scope of this invention.

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A DNA MOLECULE ENCODING A MUTANT PREPRO-NEUROPEPTIDE Y, A MUTANT SIGNAL PEPTIDE, AND USES THEREOF

FIELD OF THE INVENTION

This invention relates to a DNA sequence encoding a mutant human prepro-neuropeptide Y (preproNPY), the mutant signal peptide as such or associated with any other cleavage product of preproNPY, methods for the determination, in a biological sample, of said DNA sequence or said peptide. Furthermore, this invention relates to a method for diagnosing a predisposition for increased serum cholesterol or LDL cholesterol in a human subject, and to methods for treating a human subject diagnosed for predisposition for increased serum cholesterol or LDL cholesterol. Transgenic animals carrying either the mutant sequence or the normal sequence are also within the scope of this invention.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

Neuropeptide Y (NPY) is a 36-amino-acid peptide hormone abundantly expressed both in the central and peripheral nervous systems. NPY plays a central role in the hypothalamic regulation of food intake and energy expenditure. Central administration of NPY markedly stimulates feeding, and chronic infusion results in development of obesity, hyperinsulinemia and insulin resistance in experimental animals. Relatively little is known of the role of NPY in human obesity or metabolic diseases.

Neuropeptide Y (NPY), a member of a family of peptides, is a neurotransmitter, which is widely expressed both in the

central and peripheral nervous systems[1,2]. Several regulatory functions have been implicated to NPY including feeding[3,4,5], anxiolysis[6,7], pituitary hormone release[8,9,10], thermogenesis[11] and insulin release[12].

In animals NPY plays an important role in the hypothalamic regulation of energy balance. NPY markedly stimulates food intake after central administration[13]. It also decreases energy expenditure by decreasing brown adipose tissue thermogenesis, and favors energy storage by increasing lipoprotein lipase activity in white adipose tissue[14]. Chronic intracerebroventricular infusion of NPY results in the development of obesity and insulin resistance[13]. Food restriction markedly enhances hypothalamic NPY activity, while re-feeding decreases it, and the hypothalamic NPY neurons are controlled by peripheral hormonal feedback signals, like insulin and leptin[14,15,16]. Consequently, hypothalamic expression of preproNPY mRNA and NPY levels are elevated in obese *fa/fa* Zucker rats[17], which have impaired leptin signaling due to a point mutation in the leptin receptor gene[18]. In humans, NPY concentrations in the cerebrospinal fluid of anorexia patients are elevated[19], which is consistent with the putative compensatory activation of NPY mechanisms. Importantly anorexia patients also show elevated cholesterol levels[20,21]. However no reports were available from the literature connecting NPY gene or NPY as such to cholesterol metabolism or serum cholesterol levels.

SUMMARY OF THE INVENTION

According to one aspect, this invention concerns a DNA sequence comprising a nucleotide sequence encoding a prepro-neuropeptide Y (preproNPY) where the leucine amino acid in position 7 of the signal peptide part of said preproNPY has been replaced by proline.

According to a second aspect, the invention concerns a method for screening a subject to determine if said subject is a carrier of a mutant NPY gene, comprising the steps of providing a biological sample of the subject to be screened; and providing an assay for detecting in the biological sample the presence of i) the normal NPY gene or II) the mutant NPY gene.

According to a third aspect, the invention concerns a signal peptide having the leucine in the 7 position replaced by proline, and said signal peptide associated with any other cleavage product of preproNPY.

According to a fourth aspect, this invention concerns an antibody capable of binding said signal peptide or said signal peptide associated with any other cleavage product of preproNPY, and to an immunoassay for the determination of said peptide in a biological sample.

According to a fifth aspect, the invention concerns a method for diagnosing a predisposition for increased serum cholesterol or LDL cholesterol level in a human subject, said method comprising determining whether said subject has a polymorphism in the signal peptide part of the human preproNPY, said polymorphism comprising the subsitution of the position 7 leucine for proline in the signal peptide part of said preproNPY, said polymorphism being indicative of a predisposition to increased serum cholesterol or LDL cholesterol level.

According to a sixth aspect, the invention relates to a method for treating a human subject, diagnosed for predisposition increased serum cholesterol or LDL cholesterol level, for the prevention of increased serum cholesterol or LDL cholesterol level in said subject comprising administering to said subject an effective amount of an agent counteracting the influence of the mutated NPY gene.

According to a seventh aspect, the invention relates to a method for treating a human subject, diagnosed for predisposition of increased serum cholesterol or LDL cholesterol levels, for the prevention of increased serum cholesterol or LDL cholesterol levels in said subject comprising subjecting the person to specific gene therapy aimed to repair the mutated NPY sequence.

According to still one aspect, the invention concerns a transgenic animal which carries a human DNA sequence comprising a nucleotide sequence encoding a prepro-neuropeptide Y (preproNPY) where the leucine amino acid in position 7 of the signal peptide part of said preproNPY is i) either replaced by proline, or ii) is unchanged.

According to still one aspect, the invention concerns a transgenic animal which carries a DNA sequence comprising a nucleotide sequence encoding otherwise normal mouse NPY sequence or part thereof encoding mature mouse NPY peptide, but in which the nucleotide sequence encoding the mouse signal peptide is replaced by human signal peptide sequence encoding either normal or mutated human signal peptide.

According to still on e aspect, the invention concerns a cell line expressing the mutated human NPY gene or part thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a illustrates schematically the molecular structure of the human NPY gene, the preproNPY peptide and the mature NPY peptide,

Figure 1b shows the nucleotide sequence of the human NPY gene. Upper case indicates exonic sequences land lower case intronic sequences. Genbank accession numbers are given in parenthesis. The arrow shows the position in which T of the normal gene is replaced by C to give the mutant gene. The

underlined sequence in Exon 2 is the sequence encoding the signal peptide of 28 amino acids (Exon 1 is SEQ ID NO:1, exon 2 is SEQ ID NO:2, exon 3 is SEQ ID NO:3 and exon 4 is SEQ ID NO:4),

Figure 1c shows the nucleotide sequence of the human preproNPY mRNA (SEQ ID NO:5, with the protein sequence set forth in SEQ ID NO:6). The arrow shows the position in which t of the normal mRNA is replaced by c to give the mutant mRNA,

Figure 2 shows a) the fasting serum total cholesterol, b) LDL-cholesterol, c) HDL-cholesterol and d) VLDL-cholesterol in obese subjects, where the filled bars represent subjects (n=120) homozygous to Leu7/Leu7 of the signal peptide of the preproNPY and the empty bars represent subjects (n=21) heterozygous to Leu7/Pro7 or homozygous to Pro7/Pro7 in the signal peptide of the preproNPY, and

Figure 3 shows a) the fasting serum total cholesterol, b) LDL-cholesterol, c) HDL-cholesterol and d) VLDL-cholesterol in normal weigth subjects, where the filled bars represent subjects (n=56) homozygous to Leu7/Leu7 of the signal peptide of the preproNPY and the empty bars represent subjects (n=8) heterozygous to Leu7/Pro7 or homozygous to Pro7/Pro7 in the signal peptide of the preproNPY.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a part of the inventors' study program to investigate the genetic background of energy metabolism and obesity. We report here the identification of a rather common polymorphism in the signal peptide part of the NPY gene. Surprisingly, this Leu7 to Pro polymorphism was found to associate with significant elevation of both total and LDL cholesterol levels in normal weight and obese, non-diabetic subjects, while it was not related with energy metabolism or obesity.

The DNA sequence or the mutant signal peptide or said peptide associated with any other cleavage product of preproNPY can be used for screening a subject to determine if said subject is a carrier of a mutant NPY gene.

The determination can be carried out either as a DNA analyse according to well known methods, which include direct DNA sequencing of the normal and mutated NPY gene, allele specific amplification using the polymerase chain reaction (PCR) enabling detection of either normal or mutated NPY sequence, or by indirect detection of the normal or mutated NPY gene by various molecular biology methods including e.g. PCR- single stranded conformation polymorphism (SSCP)-method or denaturing gradient gel electrophoresis (DGGE). Determination of the normal or mutated NPY gene can also be done by using restriction fragment length polymorphism (RFLP)-method, which is particularly suitable for genotyping large number of samples.

The determination can also be carried out at the level of RNA by analysing RNA expressed at tissue level using various methods. Allele spesific probes can be designed for hybridization. Hybridization can be done e.g. using Northern blot, RNase protection assay or in situ hybridization methods. RNA derived from the normal or mutated NPY gene can also be analysed by converting tissue RNA first to cDNA and thereafter amplifying cDNA by an allele spefic PCR-method.

Alternatively, the determination can be carried out as an immunoassay where a sample is contacted with an antibody capable of binding the signal peptide or said peptide associated with any other cleavage product of preproNPY.

Antibodies can be raised against normal or mutated preproNPY or more specifically against normal or mutated signal peptide part of the NPY. The production of

antibodies can be done in experimental animals in vivo to obtain polyclonal antibodies or in vitro using cell lines to obtain monoclonal antibodies.

A human subject, diagnosed for predisposition of increased serum cholesterol or LDL cholesterol levels, can be treated for the prevention of increased serum cholesterol or LDL cholesterol in said subject by administering to said subject an effective amount of an agent counteracting the influence of the mutated NPY gene. This can be done by specific gene therapy aimed to repair the mutated NPY sequence, or by administering pharmacotherapies, which are aimed to modulate synthesis, release or metabolism of the endogenous NPY, or to interact in a specific manner at NPY target sites by modulating effects of NPY with specific NPY receptor proteins. Currently, five different subtypes of NPY receptors have been cloned and characterized (Y1-Y5 receptors) and drug molecules specifically interacting with these NPY receptors have been synthesized. The pharmacotherapy described is not limited to only these named receptors or mechanisms, but also covers other NPY receptors and related mechanisms to be discovered.

Influence of the mutated NPY sequence on the funtion of NPY gene can be investigated in transgenic animals. A transgenic animal can be generated using targeted homologous recombination methodology. Both normal and mutated sequence of human NPY signal peptide (or any DNA sequence comprising a nucleotide sequence encoding a prepro-neuropeptide Y (preproNPY) or part thereof encoding the amino acid sequence of the mature mouse or human mature NPY peptide, where either i) the leucine amino acid in position 7 of the signal peptide part of said preproNPY has been replaced by proline or ii) the leucine amino acid in position 7 of the signal peptide part of said preproNPY is unchanged) will be introduced into the sequence of NPY gene to replace the endogenous signal peptide sequence. Under these conditions, the endogenous NPY gene functions

otherwise normally, but the synthesis of the preproNPY is regulated by either normal or mutated human NPY signal peptide sequence. This transgenic model can be used to investigate in a very specific manner the physiological importance of the mutated NPY gene. It also will provide an ideal preclinical model to investigate and screen new drug molecules, which are designed to modify the influence of the mutated NPY gene.

The invention is described more in detail in the following experiments.

EXPERIMENTS

METHODS

Coding regions of the NPY gene were screened for possible sequence variants in 90 Finnish obese subjects using the single-stranded conformation polymorphism (SSCP)- analysis. Allelic associations of the identified Leu7 to Pro polymorphism with obesity-related and metabolic parameters were analyzed in two independent study populations after genotyping 141 obese, non-diabetic subjects (study I) and 64 normal weight subjects (study II) using the restriction length polymorphism (RFLP) method.

Study subjects for SSCP screening of the NPY gene

The DNA samples from 90 randomly selected obese Finns of the study I population were used to screen NPY gene for exonic sequence variants.

Study subjects for association and genotype frequency analyses

Study I

141 (29 men and 112 women) obese subjects of a weight

reduction study (Uusitupa et al. 1996) with a normal liver, kidney and thyroid function were included in the association study of NPY sequence variant with phenotype parameters. None of the subjects had diabetes, history of excessive alcohol intake or taking drugs known to affect basal metabolic rate (BMR), cholesterol (except one subject that was on a betablocking agent) or glucose metabolism. Their mean ± SD age was 43 ± 8 years and the mean body mass index (BMI) 34.7, range 28-43 kg/m^2. All phenotype measurements were done in the morning after a 12-h fast by standardized methods. The measurements included weight, BMI, percental fat, respiratory quotient (RQ), BMR, waist-to-hip ratio (WHR), fasting serum leptin, glucose, insulin, cholesterol and triglyceride levels. The main characteristics of the study I subjects are presented in Table 1. The analytical methods have been described elsewhere in detail[22,23]. A diet diary was available of all obese subjects with detailed data on the daily intake of several nutrients including carbohydrate, protein, fat and cholesterol.

Study II

Originally a random control population sample, aged 45-64 years, was selected during 1979-1981 from the population registers of the Kuopio county, Finland by using random number tables, taking into account the distribution of the population living in rural and urban communities. Of 183 subjects originally contacted, finally 144 were recruited. The normal weight (BMI<27kg/m^2) subjects were selected among the control subjects for the present investigation (study II) and they were followed for 10 years. Altogether 64 (26 men and 38 women) normoglycemic, non-diabetic healthy Finns were examined. The control subjects were re-examined after 5 and 10 years from the first examination in the years 1985-1986 and 1991-1992, respectively. The main characteristics of the study II population in these respective time point are presented in Table 2. The

protocol was approved by the Ethics Committees of the University of Kuopio and Helsinki. The study II population has been described in detail previously[24].

PCR-SSCP analysis

The human NPY gene is divided into four exons, the first containing a nontranslated region, the second exon coding signal peptide (amino acid residues 1-28) and mature NPY amino acid residues 29-63, the third exon coding residues 64-90, and the fourth exon contains the carboxy terminal heptapeptide of proNPY and the nontranslated 3'- region (Figure 1a)[25]. The PCR pimer pairs and the respective PCR annealing temperatures (Ta) for amplification of the four exonic areas of the NPY gene were as follows,: Pair 1 5' TTGGGGTGTGGGTGGCTC (SEQ ID NO:7) and 5' CCTAGACAGACGGGTCGTAGCA (SEQ ID NO:8), at Ta=65°C, pair 2 5' CCCGTCCGTTGAGCC TTCTG (SEQ ID NO:9) and 5' CGGTCCCGCGGTCCC (SEQ ID NO:10) Ta=67°C, pair 3 5' AAAAGACTTTTTTT TTTCCAG (SEQ ID NO:11) and 5' AATGTCCCCATCACAAG (SEQ ID NO:12) Ta=51°C, and pair 4 5' CCTTACAT GCTTTGCTTCTTA (SEQ ID NO:13) and 5' GATTTTTCATTGAGGAGGAT (SEQ ID NO:14) at Ta=51°C. The PCR reaction (total 5 µl) contained 100 ng genomic DNA (isolated either from whole blood or immortalized lymphoblast cell lines), 1.0 mM dNTPs, 30nM ^{33}P-dCTP, 2.5 mM each primer, 0.25 U of AmpliTaq polymerase (Perkin Elmer Cetus, Norwalk, CT). PCR conditions were optimized using PCR Optimizer™ (Invitrogen, San Diego, CA). Samples were amplified with a GeneAmp PCR System 9600 (Perkin Elmer Cetus, Norwalk, CT), 30 cycles consisting for 30 sec at 94°C, 30 sec at optimal annealing temperature and 30 sec at 72°C. This was followed by an elongation step 7 min at 72°. The amplified samples were mixed with SSCP buffer containing 95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromphenol blue (total volume of 25 µl). Prior to loading, samples were denatured 5 min at 95° C and kept 5

min on ice. Three µl of the mixture was loaded on a MDE™ gel (FMC, BioProducts, Rockland, MA). The SSCP-gel electrophoresis was performed at two different running conditions: 6 % MDE gel at +4°C and 3 % MDE gel with 10% glycerol at room temperature. Electrophoresis was run at 5 W constant power for 20 hr. The gel was dried and autoradiography was performed by exposing a Kodak BIO MAX MR film for 24 hours at room temperature.

Sequencing

The abnormally migrating bands in SSCP were sequenced with the Thermo Cycle Sequenase™ kit (Amersham Life Science, Inc. Cleveland, OH).

Genotyping

The primers used for genotyping of subjects in study I and II were those used for the exon 2 PCR amplification. In the exon 2 the T(1128) to C(1128) substitution generates an Bsi E I (New England Biolabs, Inc. Beverly, MA) site. Digestions were analyzed by electrophoresis in 2% agarose gel.

Fasting serum parameters and anthoropometric measurements

Blood glucose was analyzed by the glucose-oxidase method (Glox: Kabi Ab, Stockholm, Sweden). Serum insulin was analyzed by radioimmunoassay (antiserum M 8309: Novo Industries, Copenhagen, Denmark). The variation coefficient of the method was 5.4%, and the sensitivity was 2 mU/l. Serum and lipoprotein lipids were determined from 12-h fasting samples. Lipoproteins were separated by ultracentrifugation at density 1.006 to remove VLDL, followed by precipitation of the infranatant fraction by dextran sulphate and magnesium chloride[26]. Enzymatic methods were used for the determination of cholesterol[27] and triglycerides[28] from whole serum, the top layer after

ultracentrifugation of VLDL, and the supernatant after precipitation of LDL. LDL was calculated as the difference between whole serum and the sum of VLDL and HDL. The intra-assay variation for total cholesterol, HDL cholesterol, and triglycerides was 1.3%, 0.95%, and 3.1%respectively, and the interassay variation was 3.3%, 1.9%, and 5.2%, respectively. Standing height was measured without shoes to the nearest 0.5 cm. Body weight was measured with an electric weighing machine (model 707: Seca. Hamburg, Germany) with the subjects barefoot and dressed in shorts. Body mass index (BMI) was calculated (body weight[kg]/height[m^2]). For the waist /hip ratio the waist circumference was measured at the level of the midway between the lateral lower rib margins and the iliac crest. Hip circumference was measured at the level of the greater trochanters trough the pubic symphysis. Resting energy expenditure was measured by indirect calorimetry (Deltatrac, TM Datex, Helsinki, Finland) using a computerterized flow-through, canopy-gas analyzer system, which was calibrated with the precision gas mixture before each measurement. The method is described previously in detail[29].

Statistical analysis

The genotype frequency distribution was tested for Hardy-Weinberg equilibrium by X^2-analysis. All calculations concerning the association analysis were performed using the SPSS/WIN program version 6.0 (SPSS, Chicago, IL). Statistical differences in phenotype parameters between the two groups were evaluated using the Student's t test. In study I multiple comparisons between the genotype and phenotype parameters were done without a formal correction for multiple testing. In the study II, we had an *a priori* hypothesis that the polymorphism associates with serum cholesterol level, and therefore no other statistical comparions were carried out than that of fasting serum total, LDL, HDL and VLDL cholesterol levels.

RESULTS

The SSCP screening resulted in detection of thymidine(1128) to cytosine substitution leading to leucine to proline amino acid change at the residue 7, of the hydrophobic signal peptide part of the preproNPY. The allele frequency of the Leu7 to Pro polymorphism was 0.08 for both normal weight and obese subjects. The obese subjects having the Pro7 allele had significantly higher fasting serum total, LDL and VLDL cholesterol levels and lower HDL cholesterol level, when compared to corresponding values in subjects with the Leu//Leu7 genotype. The respective values were 6.2 ± 1.1 vs. 5.3 ± 0.9 mmol/l (P=0.0001), 4.2 ± 1.0 vs. 3.5 ± 0.8 mmol/l (P=0.0003), 0.9 ± 0.6 vs. 0.7 ± 0.5 mmol/l (P=0.042) and 1.1 ± 0.3 vs. 1.2 ± 0.3 mmol/l (P=0.041). These differences could not be explained by confounding factors including age, sex, smoking, concomitant medication or the apoE -phenotypes. The Leu7 to Pro polymorphism in the NPY gene did not associate with any obesity related parameter including weight, BMI, waist-to-hip ratio, fat mass, basal metabolic rate or other metabolic parameters such as fasting plasma levels of glucose, insulin, leptin or triglycirides in obese subjects. The significant association of the Pro7 allele with higher serum total cholesterol (p=0.035) and LDL- cholesterol levels (p=0.036) was confirmed in normal weight subjects of the study II.

SSCP screening of the exonic areas of the NPY gene

Individual exons comprising the whole coding region of the NPY gene were screened for mutations by SSCP. The identified polymorphism were 1)T(1128) to C(1128), 2)A(1258) to G(1258), 3)T(5671) to C(5671), and 4)T(8233) to A(8233). The numbering of the polymorphism is according to the Minth et al. 1986, in which the polymorphism 2 and 3 were already reported[25].

Genotype frequencies

All the allelic frequencies are in Hardy-Weinberg equilibrium. The allele frequency of the found T(1128) to C(1128) polymorphism were 0.078 in obese (n=141) and 0.077 in normal weight control Finns (n=64). There were no differences in any of the allelic distributions between these two populations.

Association analysis

Study I:

The homozygote Pro7/Pro7 genotype was detected in only one subject, who was included to the heterozygote group. The association analysis between the Pro7/Leu7 genotype subjects (including one Pro7/Pro7 genotype) and the wild type Leu7/Leu7 genotype subjects revealed highly significant differences in fasting serum total cholesterol 6.2 ± 1.1 vs. 5.3 ± 0.9 mmol/l (P=0.0001), LDL cholesterol 4.2 ± 1.0 vs. 3.5 ± 0.8 mmol/l (P=0.0003), and VLDL cholesterol 0.9 ± 0.6 vs. 0.7 ± 0.5 mmol/l (P=0.042) levels and HDL cholesterol 1.1 ± 0.3 vs. 1.2 ± 0.3 mmol/l (P=0.041) (Figure 2). The differences remained highly significant if the analysis was performed separately in obese men (total-cholesterol, LDL cholesterol, VLDLcholesterol and HDL cholesterol) and in obese women (total cholestrol and LDL cholesterol). The intake of total fat, saturated fatty acids, unsaturated fatty acids or dietary cholesterol did not differ in the two genotype group. The degree of obesity does not explain these findings, either. There were no differences in the distribution of apolipoprotein-E phenotypes between the different groups (data not shown).

Study II:

One subject was homozygote Pro7/Pro7 and was analyzed together with the heterozygotes. In normal weight subjects the fasting serum total and LDL cholesterol levels were

significantly higher in subjects having the Pro7 allele than in subjects with the Leu7/Leu7 genotype of every three measurement. Fasting serum total cholesterol 7.4 ± 0.6 vs. 6.7 ± 0.9 mmol/l (P=0.035), LDL cholesterol 5.2 ± 0.6 vs. 4.5 ± 0.9 mmol/l (P=0.036). There were no statistically significant differences in VLDL cholesterol (0.8 ± 0.5 vs. 0.7 ± 0.4 mmol/l) or HDL cholesterol (1.3 ± 0.4 vs. 1.5 ± 0.3 mmol/l levels) (Figure 3).

DISCUSSION

The present study provides the first evidence that the Leu7 to Pro polymorphism in NPY gene associates with clinically unfavorable serum cholesterol and LDL cholesterol levels both in normal weight and non-diabetic obese subjects. This indicates that NPY may have a previously unrecognized role in the regulation of cholesterol metabolism in human and is one of the strongest genetic factors thus far identified affecting serum cholesterol levels.

The major observation of the present study is that the identified polymorphism leucine7 to proline in the signal peptide part of the NPY gene significantly associates with elevated serum total and LDL cholesterol levels in Finns. Furthermore, in obese subjects also VLDL cholesterol was significantly increased and HDL cholesterol decreased in subjects with the Pro7 allele. The main finding was initially done in obese, non-diabetic subjects, and was subsequently repeated in normal weight subjects. The allele frequency of this sequence variant was about 8 % in the Finnish populations. The observed association cannot be explained by other confounding factors known to affect cholesterol metabolism, such as age, obesity, sex, smoking, drugs or the apoE phenotype. Furthermore, it is also highly unlikely that the association could be due to a stratification error in the study subjects, since they all were native Finns with rather similar genetic background. Thus, leucine7 to proline polymorphism of the NPY gene

should be considered as an important new genetic marker for high serum total cholesterol and LDL cholesterol levels.

The leucine7 to proline polymorphism is located in the signal peptide part of the preproNPY. The signal peptide, which is cleaved away from the mature NPY, plays an important role by guiding proper folding and packing of the peptide in the endoplasmic reticulum during the synthesis and transport into secretatory vesicles. Usually the signal peptide consists of a hydrophobic motif as is the case with preproNPY. Leucine is known to form α-helices, while proline usually introduces breaks and kinks into α-helical parts of the peptide backbone. Although we do not have biochemical data how the leucine7 to proline polymorphism modifies the synthesis of the preproNPY, one could speculate that intracellular processing of preproNPY synthesis is impaired, which subsequently could lead to altered NPY activity. However, further studies are required to elucidate these mechanisms in detail.

Serum total cholesterol and LDL cholesterol levels were on average 0.9 and 0.7 mmol/l, respectively, higher in obese and non-obese Finnish subjects having the Pro7 allele compared to those having Leu7/Leu7 genotype. Moreover, a trend to a higher VLDL cholesterol and lower HDL cholesterol were found in these subjects. The impact of this genetic abnormality on serum cholesterol level is greater than that of apo E 4 allele[30], and is of the same magnitude (14%) that could be obtained at best by cholesterol lowering diet therapy in free living Finnish subjects.

What are then the reasons for the elevation of serum total and LDL cholesterol in these subjects representing of 8% of Finnish population? Due to fact that gastrointestinal tract is abundantly innervated by NPY containing nerves[31,32] one can speculate that NPY could be involved in the absorption of dietary cholesterol, and subjects with the Pro7 allele

might have an increased cholesterol absorption. This, on the other hand, could result in down-regulation of B/E (LDL) receptor activity of the liver and an elevation of LDL and its precursors in serum, e.g. VLDL. Because there were no marked abnormalities in VLDL or triglyceride levels in the affected subjects we consider that the primary defect can not be in the synthesis or the catabolism of VLDL. Interestingly, however, central NPY increases the expression of lipoprotein lipase mRNA and enhances the enzyme activity in white fat favoring lipid storage. Therefore, the role of lipoprotein lipase activity can not be totally excluded. The most plausible explanation for the elevation of serum cholesterol levels is, however, diminished amount of activity of LDL receptors which are known to regulate the serum concentration of LDL, and to a lesser degree, of IDL and VLDL particles as well. Obesity as such does not seem to modify the impact of the leucine7 to proline polymorphism on serum lipids since the differences in lipid values between the mutated and normal subjects were similar in obese and normal weight subjects. After all, it should be noticed that there is no experimental evidence to support any of these mechanisms discussed above which could link this particular genetic abnormality in NPY to cholesterol metabolism.

As said before ApoE-phenotype 4 is also known to associate with higher serum total cholesterol and LDL-cholesterol levels, which has previously reported in our study subjects[22]. The apoE-phenotype 4 was evenly distributed in both NPY groups and does not confound the association of the NPY signal peptide polymorphism with differences in serum cholesterol levels.

The identified leucine7 to proline polymorphism in the NPY gene did not seem to associate in the present study to any obesity related parameters, like weight, BMI, WHR, BMR or RQ. In agreement, the allele frequencies of the mutated allele were similar in normal weight controls and obese,

non-diabetic subjects. This result is also consistent with a recent study performed in a French population, in which flanking markers of the NPY gene failed to be in linkage with any traits of obesity[33].

The present study provides the first evidence that the leucine7 to proline polymorphism in NPY gene associates with clinically unfavorable serum cholesterol and lipoprotein levels both in non-diabetic normal weight and obese subjects. This indicates that NPY may have a previously unknown role in the control of cholesterol metabolism in man and is one of the strongest genetic factors thus far identified affecting serum cholesterol levels. Furthermore, NPY mechanisms could offer potential targets to the development of new drugs.

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

Table 1. Demographic and clinical characteristics of 141 obese subjects according to the presence or absence of the Leu(7) to Pro(7) mutation in the NPY gene. The values are mean ± SD.

Characteristic	Without mutation	With mutation	P value
age, years	40,7±6.2	41.2±8.1	ns
sex, F/M	95/25	17/4	ns
BMI, kg/m^2	34.7±3.8	35.7±3.3	ns
WHR	0.93±0.08	0.94±0.08	ns
BMR, kcal/d*	1635±142	1639±131	ns
fs-insulin, pmol/l	94.8±45.3	97.7±53.5	ns
fs-glucose, mmol/l	5.5±0.7	5.5±0.8	ns
fs-leptin, ng/l**	32.9±12.8	26.3±4.9	ns
Systolic blood pressure, mmHg	130.7±14.8	128.6±13.2	ns
Diastolic blood pressure, mm Hg	87.4±10.8	84.7±6.6	ns

*Adjusted for fat free mass and age. **The leptin levels were available from 69 subjects.

Table 2. Demographic and clinical characteristics of 64 normal weight subjects in the beginning of the follow-up study (during 1979-1981) according to the presence or absence of Leu(7) to Pro(7) mutation in the NPY gene. The values are mean ± SD

Characteristic	Without mutation	With mutation
age, years	55.8±2.0	55.1±1.8
sex, F/M	31/25	7/1
BMI, kg/m^2	24.3±2.0	24.9±1.8
WHR	0.87±0.08	0.85±0.05
fs-insulin, pmol/l	65.4±44.4	84.0±42.6
fs-glucose, mmol/l	4.9±0.63	4.5±0.57
Systolic blood pressure, mmHg	142.4±17.6	151.1±16.1
Diastolic blood pressur , mm Hg	86.8±9.0	91.1±8.4

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Koulu, Markku
Karvonen, Matti
Pesonen, Ullamari
Uusitupa, Matti

(ii) TITLE OF INVENTION: A DNA Molecule Encoding a Mutant Prepro-Neuropeptide Y, a Mutant Signal Peptide, and Uses Thereof

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/994,946
(B) FILING DATE: 19-DEC-1997
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 28,957
(C) REFERENCE/DOCKET NUMBER: 2328-110

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(B) TELEFAX: 202-783-6031

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 325 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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CCAAGCCGGA CAACCCGGGC GAGGACGCAC CAGCGGAGGA CATGGCCAGA TACTACTCAG 180

CGCTGGGACA CTACATCAAC CTCATCACCA GGCAGAGGTG GGTGGGACCG CGGGACCGAT 240

TCCGGGA 247

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(A) LENGTH: 142 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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GGCTTGTGAT GGGGACATTG TT 142

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(A) LENGTH: 300 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(A) LENGTH: 551 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 87..170

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 87..377

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Met Leu Gly Asn Lys Arg Leu Gly Leu
1 5

TCC GGA CTG ACC CTC GCC CTG TCC CTG CTC GTG TGC CTG GGT GCG CTG 161
Ser Gly Leu Thr Leu Ala Leu Ser Leu Leu Val Cys Leu Gly Ala Leu
10 15 20 25

GCC GAG GCG TAC CCC TCC AAG CCG GAC AAC CCG GGC GAG GAC GCA CCA 209
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30 35 40

GCG GAG GAC ATG GCC AGA TAC TAC TCG GCG CTG CGA CAC TAC ATC AAC 257
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60 65 70

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75 80 85

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Arg Leu Glu Asp Pro Ala Met Trp
90 95

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(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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 1               5                  10                  15

Ser Leu Leu Val Cys Leu Gly Ala Leu Ala Glu Ala Tyr Pro Ser Lys
            20                  25                  30

Pro Asp Asn Pro Gly Glu Asp Ala Pro Ala Glu Asp Met Ala Arg Tyr
        35                  40                  45

Tyr Ser Ala Leu Arg His Tyr Ile Asn Leu Ile Thr Arg Gln Arg Tyr
    50                  55                  60

Gly Lys Arg Ser Ser Pro Glu Thr Leu Ile Ser Asp Leu Leu Met Arg
65                  70                  75                  80

Glu Ser Thr Glu Asn Val Pro Arg Thr Arg Leu Glu Asp Pro Ala Met
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Trp
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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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(2) INFORMATION FOR SEQ ID NO:10:

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(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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(2) INFORMATION FOR SEQ ID NO:12:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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(2) INFORMATION FOR SEQ ID NO:13:

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(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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(2) INFORMATION FOR SEQ ID NO:14:

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(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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CLAIMS

1. A DNA sequence comprising a nucleotide sequence encoding a prepro-neuropeptide Y (preproNPY) where the leucine amino acid in position 7 of the signal peptide part of said preproNPY has been replaced by proline.

2. The DNA sequence according to claim 1 comprising the genomic nucleotide sequence shown in Figure 1b.

3. The DNA sequence according to claim 1 wherein said DNA sequence is cDNA.

4. An RNA sequence comprising an RNA sequence corresponding to the DNA sequence of claim 1.

5. A method for screening a subject to determine if said subject is a carrier of a mutant NPY gene, comprising the steps of
- providing a biological sample of the subject to be screened; and providing an assay for detecting in the biological sample the presence of i) the normal NPY gene or II) the mutant NPY gene.

6. The method according to claim 5 where the assay is any assay utilizing the information of the DNA sequence according to claim 1.

7. The signal peptide having the leucine in the 7 position replaced by proline.

8. A peptide comprising the signal peptide according to claim 7 associated with any other cleavage product of preproNPY.

9. An antibody capable of binding the signal peptide according to claim 7.

10. An antibody capable of binding the peptide according to claim 8.

11. An immunoassay for the determination of a peptide as defined in claim 7 or 8 wherein a biological sample is exposed to an antibody capable of binding said peptide.

12. A method for diagnosing a predisposition for increased serum cholesterol or LDL cholesterol level in a human subject, said method comprising determining whether said subject has a polymorphism in the signal peptide part of the human preproNPY, said polymorphism comprising the subsitution of the position 7 leucine for proline in the signal peptide part of said preproNPY, said polymorphism being indicative of a predisposition to increased serum cholesterol or LDL cholesterol level.

13. A method for treating a human subject, diagnosed for predisposition of increased serum cholesterol or LDL cholesterol levels according to claim 12, for the prevention of increased serum cholesterol or LDL cholesterol levels in said subject comprising administering to said subject an effective amount of an agent counteracting the influence of the mutated NPY gene.

14. The method according to claim 13 wherein said agent is a pharmaceutical aimed to modulate synthesis, release or metabolism of the endogenous NPY, or to interact in a specific manner at NPY target sites by modulating effects of NPY with specific NPY receptor proteins.

15. The method according to claim 13 wherein said agent is a pharmaceutical aimed to modulate gene expression of normal or mutated NPY gene.

16. A method for treating a human subject, diagnosed for predisposition of increased serum cholesterol or LDL cholesterol levels according to claim 12, for the

prevention of increased serum cholesterol or LDL cholesterol levels in said subject comprising subjecting the person to specific gene therapy aimed to repair the mutated NPY sequence.

17. A transgenic animal which carries a human DNA sequence comprising a nucleotide sequence encoding a prepro-neuropeptide Y (preproNPY) or part thereof encoding mature human NPY peptide, where the leucine amino acid in position 7 of the signal peptide part of said preproNPY has been replaced by proline.

18. A transgenic animal which carries a human DNA sequence comprising a nucleotide sequence encoding a prepro-neuropeptide Y (preproNPY) or part thereof encoding mature human NPY peptide, where the leucine amino acid in position 7 of the signal peptide part of said preproNPY is unchanged.

19. A transgenic animal which carries a DNA sequence comprising a nucleotide sequence encoding otherwise normal mouse NPY sequence or part thereof encoding mature mouse NPY peptide, but in which the nucleotide sequence encoding the mouse signal peptide is replaced by human signal peptide sequence encoding either normal or mutated human signal peptide.

20. A cell line expressing the mutated human NPY gene or part thereof.

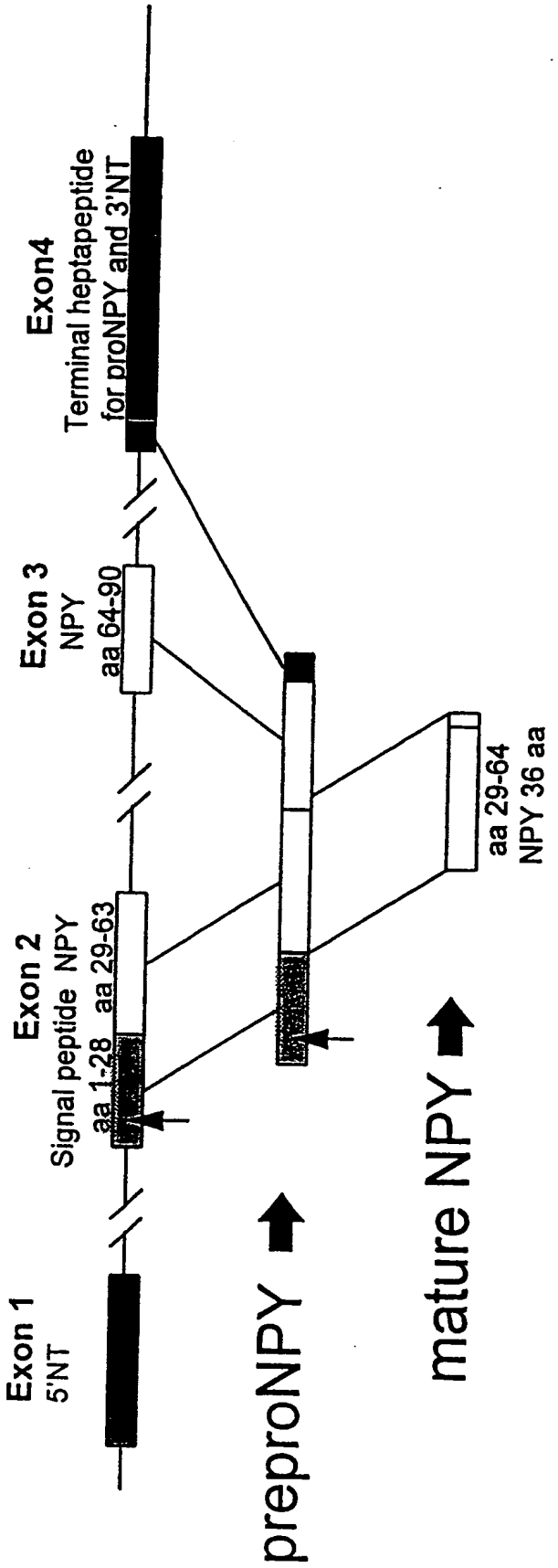
Exon4
Terminal heptapeptide
for proNPY and 3'NT
Exon 3
NPY
aa 64-90
Exon 2
Signal peptide NPY
aa 1-28 aa 29-63
Exon 1
5'NT
aa 29-64
NPY 36 aa
preproNPY
mature NPY

FIG. 1a

HUMAN NEUROPEPTIDE Y (NPY) GENE

EXON 1 (M14295)

```
1   ccgcttcttc aggcagtgcc tggggcggga gggttggggt gtgggtggct ccctaagtcg
61  acactcgtgc ggctgcggtt ccagcccccc ccccccgcca ctcaggggcg ggaagtggcg
121 ggtgggagtc acccaagcgt gactgcccga ggcccctcct gccgcggcga ggaagctcca
181 taaaagccct gtcgcgaccc gctctctgcA CCCCATCCGC TGGCTCTCAC CCCTCGGAGA
241 CGCTCGCCCG ACAGCATAGT ACTTGCCGCC CAGCCACGCC CGCGCGCCAG CCACCGTGAG
301 tgctacgacc cgtctgtcta ggggt
```

EXON 2 (M14296)

C ↓

```
1   cccgtccgtt gagccttctg tgcctgcagA TGCTAGGTAA CAAGCGACTG GGGCTGTCCG
61  GACTGACCCT CGCCCTGTCC CTGCTCGTGT GCCTGGGTGC GCTGGCCGAG GCGTACCCCT
121 CCAAGCCGGA CAACCCGGGC GAGGACGCAC CAGCGGAGGA CATGGCCAGA TACTACTCAG
181 CGCTGGGACA CTACATCAAC CTCATCACCA GGCAGAGgtg ggtgggaccg cgggaccgat
241 tccggga
```

EXON 3 (M14297)

```
1   acttgcttta aaagactttt ttttttccag ATATGGAAAA CGATCTAGCC CAGAGACACT
61  GATTTCAGAC CTCTTGATGA GAGAAAGCAC AGAAAATGTT CCCAGAACTC Ggtatgacaa
121 ggcttgtgat ggggacattg tt
```

EXON 4 (M14298)

```
1   CCTTACATGC TTTGCTTCTT ATGTTTTACA Ggcttgaaga ccctgcaatg tggtgatggg
61  aaatgagact tgctctctgg ccttttccta ttttcagccc atatttcatc gtgtaaaacg
121 agaatccacc catcctacca atgcatgcag ccactgtgct gaattctgca atgttttcct
181 ttgtcatcat tgtatatatg tgtgtttaaa taaagtatca tgcattcaaa agtgtatcct
241 cctcaatgaa aaatctatta caatagtgag gattattttc gttaaactta ttattaacaa
```

FIG. 1b

HUMAN NEUROPEPTIDE Y (NPY) mRNA

K01911

C

```
1   accccatccg ctggctctca cccctcggag acgctcgccc gacagcatag tacttgccgc
61  ccagccacgc ccgcgcgcca gccaccatgc taggtaacaa gcgactgggg ctgtccggac
121 tgaccctcgc cctgtccctg ctcgtgtgcc tgggtgcgct ggccgaggcg tacccctcca
181 agccggacaa cccgggcgag gacgcaccag cggaggacat ggccagatac tactcggcgc
241 tgcgacacta catcaacctc atcaccaggc agagatatgg aaaacgatcc agcccagaga
301 cactgatttc agacctcttg atgagagaaa gcacagaaaa tgttcccaga actcggcttg
361 aagaccctgc aatgtggtga tgggaaatga gacttgctct ctggcctttt cctattttca
421 gcccatattt catcgtgtaa aacgagaatc cacccatcct accaatgcat gcagccactg
481 tgctgaattc tgcaatgttt tcctttgtca tcattgtata tatgtgtgtt taaataaagt
541 atcatgcatt c
```

FIG. 1c

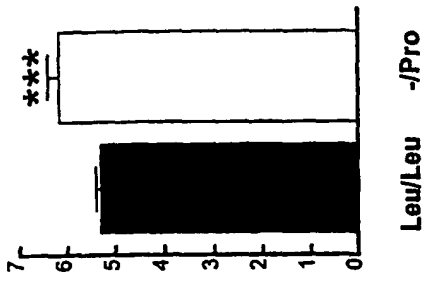

Fig. 2a

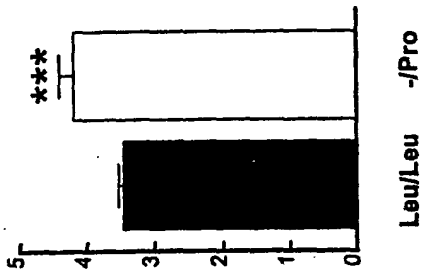

Fig. 2b

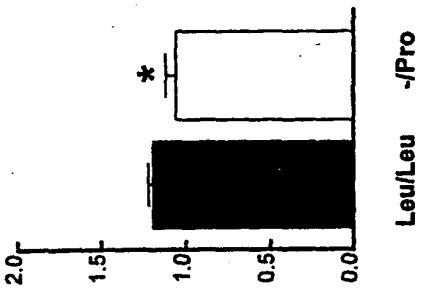

Fig. 2c

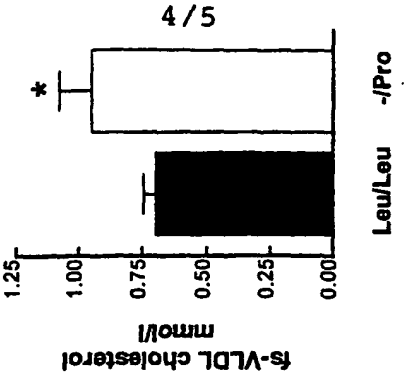

Fig. 2d

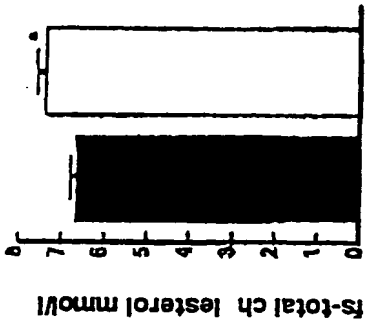

Fig. 3a

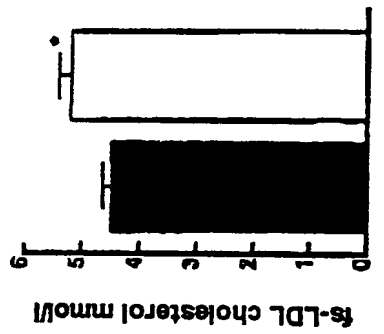

Fig. 3b

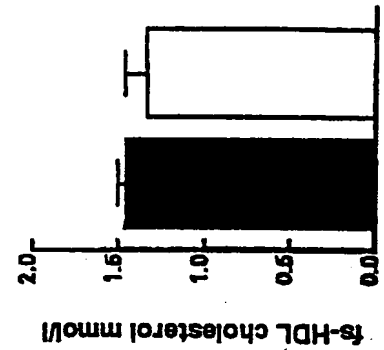

Fig. 3c

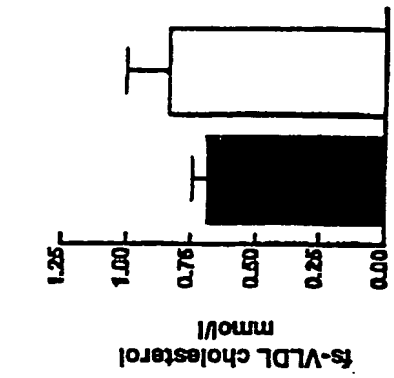

Fig. 3d

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00985

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/575, A01K 67/027, C12N 15/62
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, MEDLINE, EMBASE, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Ann Med, Volume 30, 1998, Matti I J Uusitupa et al, "Neuropeptide Y: a novel link between the neuroendocrine system and cholesterol metabolism" page 508 - page 510 --	1-17,20
P,X	Nature Medicine, Volume 4, No 12, December 1998, Matti K. Karvonen et al, "Association of a leucine(7)-to-proline(7)polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels" page 1434 - page 1437 --	1-17,20

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 April 1999	26 -04- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Hampus Rystedt Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00985

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9850563 A1 (PPL THERAPEUTICS (SCOTLAND) LTD.), 12 November 1998 (12.11.98), See claims 1,6 and 16	18
P,A		17,19,20
	--	
X	Diabetologia, Volume 40, 1997, C. Roche et al, "Genetic studies of neuropeptide Y and neuropeptide Y receptors Y1 and Y5 regions in morbid obesity", page 671 - page 675, See page 672, last paragraph	5
	--	
X	Regulatory Peptides, Volume 61, 1996, Annika Thorsell et al, "Cationic lipid-mediated delivery and expression of prepro-neuropeptide Y cDNA after intraventricular administration in rat: feasibility and limitations", page 205 - page 211, See abstract	5,18
A		6,17,19,20
	--	
X	US 5455164 A (JEFFREY D. TURNER), 3 October 1995 (03.10.95), See claims	18
	--	
A	WO 9527782 A1 (PPL THERAPEUTICS (SCOTLAND) LTD), 19 October 1995 (19.10.95)	17-20
	--	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00985

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13-16
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 13-16 relate to methods for treatment of the human body, the search has been carried out based on the alleged effects of the mutated NPY-gene.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet

1. [X] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

The application relates to four separate inventions, namely:

1. Nucleic acid sequences encoding a pre-pro-neuropeptide Y in which the leucine of position 7 in the signal peptide has been exchanged for a proline, according to claims 1-4, 7-17 and 20. The encoded peptide and its uses are also included in the invention.

2. A method for screening for mutant pre-pro-neuropeptide Y, not restricted to the mutation of invention 1, according to claims 5 and 6.

3. A transgenic animal expressing wild-type pre-pro-neuropeptide Y, according to claim 18.

4. A transgenic animal carrying a hybrid DNA-sequence encoding mouse neuropeptide Y and the human signal peptide of pre-pro-neuropeptide Y, according to claim 19.

The technical feature characterising invention 1 is the appearance and utilization of the mutation Leu→Pro in position 7 of the signal peptide. The method of invention 2 is characterised by the detection of any mutation in the neuropeptide Y gene. Invention 3 is characterised by the absence of the mutation according to invention 1. The animal according to invention 4 seems to be characterised by the joining of mouse neuropeptide Y and a human signal peptide. These four inventions are not considered to share a special technical feature as required by PCT Rule 13.

It is considered possible to search inventions 3 and 4 together within one additional fee; invention 2 requires a separate search and thus one additional fee.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/03/99

International application No.
PCT/FI 98/00985

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9850563 A1	12/11/98	AU 7224498 A	27/11/98
		GB 9708918 D	00/00/00
US 5455164 A	03/10/95	US 5227301 A	13/07/93
WO 9527782 A1	19/10/95	AU 2112095 A	30/10/95
		CA 2187173 A	19/10/95
		EP 0753059 A	15/01/97
		GB 9406974 D	00/00/00
		JP 10502243 T	03/03/98
		NZ 283150 A	27/05/98

34 SEX-SPECIFIC GENETIC INFLUENCES ON THE COMORBIDITY OF ALCOHOLISM AND MAJOR DEPRESSION

C. Prescott, S. Aggen, K. Kendler. Virginia Institute for Psychiatric and Behavioral Genetics, Medical College of Virginia of Virginia Commonwealth University, Richmond, Virginia, USA

Alcoholism and depression frequently co-occur but the origins of this comorbidity remain uncertain. We studied the importance of genetic influences on risk for lifetime comorbidity of major depression and alcoholism using a population-based twin sample. Lifetime major depression (MD), alcohol abuse, and alcohol dependence (AD) were assessed by structured interviews of both members of 3,755 twin pairs from the Mid-Atlantic Twin Registry. Individuals with MD were at significantly increased risk for AD and for a combined diagnosis of alcohol abuse and/or dependence (AAD). History of MD in a twin significantly increased risk for cotwin AD and AAD among identical male pairs and for AAD in identical female pairs, but not among male, female, or opposite-sex fraternal pairs. Results of structural modeling indicate that the familial transmission of major depression and alcohol dependence was largely disorder-specific. Comorbidity appears to be due to sex-specific genetic and environmental risk factors. There was little evidence that the factors underlying depression in women arise from the same factors as alcoholism in men.

35 POLYMORPHISM OF THE NEUROPEPTIDE Y GENE: AN ASSOCIATION STUDY WITH ALCOHOL WITHDRAWAL

T. Okubo, S. Harada
Institute of Community Medicine, University of Tsukuba, Tsukuba 3058575, Japan.

Neuropeptide Y (NPY) in both central and peripheral nervous systems modulate feeding behavior, anxiety associated behavior, circadian rhythm, seizure, and secretion of other hormones. The human NPY gene locates in Chromosome 7pter-q22. Recent studies have revealed that NPY-deficient mice consumed much amount of ethanol compared to wild type mice. In the present study, we analyzed the whole coding region and 5'-untranslating region of the NPY gene for 131 Japanese male alcoholics with different withdrawal symptoms (93 delirium tremens; 49 hallucination, 38 seizure), and 98 Japanese male controls. All subjects tested were the *ALDH2*1* homozygous genotype. Three polymorphic variants, namely -121C/A (promoter), 1258G/A (exon2) and 5671C/T (exon3) were detected in both groups. A substitution C to A in the -121 locus produces a putative binding site of GATA-1. Also, -90G/A (promoter) and 5642-5651 mononucleotide repeats T(10)/T(11) (intron2) were found as rare variants. Polymorphism(C/T) at 1128C/T locus has been reported to be associated with a higher serum cholesterol level in the obese Caucasians. But such a polymorphism was not found in our samples. The genotypic distributions for these polymorphic loci; -121C/A, 1258G/A and 5671C/T were not significantly different between the alcoholics and controls. However, frequencies of the C and T alleles were significantly different between patients with seizure and those without seizure ($p<0.03$). Consequently, our data suggested that a C to T transition at the 5671 locus of the NPY gene may associate with seizure in alcohol withdrawal.

36 NEUROPEPTIDE Y POLYMORPHISM AND ALCOHOL CONSUMPTION IN MIDDLE-AGED MEN

J.Kauhanen, M.K.Karvonen*, U.Pesonen*, M.Koulu*, T-P. Tuomainen, M.I.J.Uusitupa, J.T.Salonen. Department of Public Health and General Practice, Research Institute of Public Health, and Department of Clinical Nutrition, University of Kuopio, Box 1627, FIN-70211 Kuopio, Finland.
*Department of Pharmacology and Clinical Pharmacology, University of Turku, FIN-20520 Turku, Finland.

Neuropeptide Y (NPY) plays an important role in the hypothalamic regulation of food intake and energy balance. According to recent findings in animals, NPY also appears to be a potent regulator of alcohol consumption. We used recently identified Leu (7) to Pro (7) polymorphism in the signal peptide part of NPY to investigate wheter the NPY system is associated with alcohol consumption in humans. The subjects (N=889) were an ethnically homogenous, unselected population sample of middle-aged men from Eastern Finland. The gene variant producing Pro (7) substitution was associated with a 34 % higher average alcohol consumption, even following adjustment for a number of covariates ($p=0.03$). The proportion of heavy drinkers (over 230 grams of ethanol/week) was also somewhat higher in this group (13.1 % vs. 8.2 %, $p=0.10$). Our study provides the first evidence that alcohol preference in humans is likely to be regulated by the NPY system.

37 EVIDENCE OF LINKAGE DISEQULIBRIUM IN THE HAD2 /LAD2 RAT LINES FOR QTLS IDENTIFIED IN THE HAD1/LAD1 RAT LINES

L. Carr, T. Foroud, P. Bice, L. Lumeng, P. Castelluccio,, R. Bo, A. Ritchotte, T.-K. Li. Indiana University School of Medicine, Indianapolis, IN 46202

Selective breeding has been employed to develop replicate high-alcohol-drinking (HAD1 and HAD2) and low- alcohol-drinking (LAD1 and LAD2) rat lines from the heterogeneous N/Nih rat. A genome scan was performed in the noninbred HAD1/LAD1 lines and four chromosomal regions (5, 10, 12, and 16) were identified with lod scores greater than 2.4. Genotyping was performed in these regions in the HAD2 and LAD2 selected lines to determine if there was evidence of linkage disequilibrium. Markers were genotyped in 32 rats, 16 from the 31st selectively bred generation of the HAD2 and 16 from the 31st selectively bred generation of the LAD2. Linkage disequilibrium was defined as fixation (homozygosity) of different marker alleles in the selectively bred HAD2 and LAD2 rats. We observe evidence of linkage disequilibrium in the HAD2 and LAD2 for the marker at the QTL peak on chromosome 5 (D5mgh17) and the marker at the QTL peak on chromosome 10 (D10mgh25). While we do not observe significant evidence of linkage disequilibrium on chromosomes 12 and 16 in the QTL region, this should not be interpreted as failure to replicate the QTL finding, because linkage disequilibrium, is typically only observed a few centimorgans from a QTL. These findings suggest that at least 2 QTLs identified in the HAD1/LAD1 lines will be confirmed when the HAD2/LAD2 F2 sample is analyzed. (AA10707)

38 QUANTITATIVE TRAIT LOCI INFLUENCING cAMP SIGNALING AND SENSITIVITY TO THE INCOORDINATING EFFECTS OF ETHANOL.

S.L. Kirstein, K.L. Davidson*, V.G. Erwin*, and B. Tabakoff, Department of Pharmacology, *Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, CO 80262.

In previous work, we identified a genetic correlation between isoproterenol-stimulated cAMP accumulation in the cerebellum (CBL) and sensitivity to the incoordinating effects of ethanol. A genetic correlation suggests that common genes underlie the phenotypes investigated. One method for provisionally identifying genes potentially involved in a given phenotypic measure is quantitative trait loci (QTL) analysis. We conducted QTL analyses of initial sensitivity and acute functional tolerance (AFT) on the dowel test for ataxia and of cAMP signaling in the CBL using a panel of 32 BXD recombinant inbred strains and the progenitors (DBA/2J and C57Bl/6J). Initial sensitivity was defined by the blood ethanol (EtOH) concentration (BEC_0) at the loss of balance on a dowel (dose = 1.75 g/kg EtOH, i.p.). When mice regained balance on the dowel, BEC_1 was determined and a second EtOH injection was given (2 g/kg, i.p.). Upon final regain of balance, BEC_2 was determined. AFT was defined as the difference between BEC_1 and BEC_2 (ΔBEC). Genome-wide QTL analyses correlating strain means with allelic status at >2000 markers identified several significant associations ($p<0.01$). Provisional QTL were identified for initial sensitivity on 6 chromosomes, AFT on 9 chromosomes, and cAMP signaling on 13 chromosomes. Two loci were found to be similar for measures of initial sensitivity and for cAMP signaling. These results suggest that although ethanol sensitivity and cAMP signaling are determined by multiple genes, they may share certain genetic codetermination. Specific chromosomal map locations will be presented on the poster and data on candidate genes is being pursued by biochemical methods.

Supported by NIAAA and the Banbury Foundation.

39 GREATER INITIAL SENSITIVITY TO ETHANOL IN ETHANOL-PREFERRING sP THAN –NON PREFERRING sNP RATS

R. Agabio[1], M.A.M. Carai[1,2], C. Lobina[1,2], M. Pani[1], R. Reali[2], G. Vacca[1,2], G.L. Gessa[1,2], G. Colombo[1]
[1]Department of Neuroscience, University of Cagliari, [2]Neuroscienze S.c.ar.l., Cagliari, Italy.

The present study investigated the initial sensitivity to the ataxic and sedative/hypnotic effects of ethanol, pentobarbital and diazepam in selectively bred ethanol-preferring sP and –non preferring sNP rats. After the acute administration of 3.0 and 3.5 g/kg ethanol (i.p.), sP rats took significantly shorter times to lose the righting reflex (onset) and regained this reflex (sleep time) over significantly longer periods and at significantly lower blood ethanol levels than sNP rats. Accordingly, acute ethanol (2.0, 2.5 and 3.0 g/kg, i.g.) affected motor coordination (measured as the rat ability to run on a revolving drum) to a significantly greater extent in sP than sNP rats. In contrast, sP and sNP rats were not differentially sensitive to the sedative/hypnotic effects induced by pentobarbital (40 mg/kg; i.p.) and diazepam (15 and 20 mg/kg; i.p.). These results a) demonstrate that sP rats possess a genetically determined, greater sensitivity to the motor impairing and sedative/hypnotic effects of ethanol than sNP rats, and b) feature sP rats as an experimental model of those subsets of human alcoholics with initial high sensitivity to ethanol challenges. Furthermore, the results of the present study, together with those indicating a greater sensitivity to the sedative/hypnotic effects of gamma-hydroxybutyric acid (GHB) in sP than sNP rats (Alcohol Alcohol. 33:121-125, 1998), suggest that the brain GHB receptor system, rather than the $GABA_A$ receptor complex, may have a role in the predisposition to ethanol sensitivity of sP rats.

Alcoholism: clinical and experimental research. 24(2000): suppl.5.

Alcohol Clin Exp Res 1999 Jun;23(6):1023-30

Innate differences of neuropeptide Y (NPY) in hypothalamic nuclei and central nucleus of the amygdala between selectively bred rats with high and low alcohol preference.

Hwang BH, Zhang JK, Ehlers CL, Lumeng L, Li TK.

Department of Anatomy, School of Medicine, Indiana University, Indianapolis, 46202, USA. hwang@anatomy.iupui.edu

BACKGROUND: Neuropeptide Y (NPY) is a neuropeptide that has been demonstrated to produce anxiolytic effects when administered centrally. To examine the hypothesis that NPY might play a role in alcohol-seeking behavior, this study took advantage of the genetic differences of the alcohol-preferring (P) rats and alcohol-nonpreferring (NP) rats, as well as the high alcohol-drinking (HAD) rats and low alcohol-drinking (LAD) rats, in voluntary alcohol consumption to examine if NPY neurons in the brains differ between these selected lines. METHODS: The NPY immunoreactivity (NPY-I) was measured using an established radioimmunohistochemical assay in discrete brain structures including the paraventricular hypothalamic nucleus (PVN), arcuate nucleus (ARC), and central nucleus of the amygdala (CeA). RESULTS: The quantitative data indicated that there was more NPY-I in the PVN and ARC of P rats than NP rats, whereas there was less NPY-I in the PVN and ARC of HAD rats than LAD rats. However, the NPY-I in the CeA was less in both the P and HAD rats than in the NP and LAD rats, respectively. Therefore, the data indicate that there are innate differences in the NPY-I in the brain between selectively bred rats with high and low alcohol preference. CONCLUSION: Because both P rats and HAD rats have high alcohol preference, the disparate finding between these two lines of rats suggests that the hypothalamic NPY neurons are probably not associated with alcohol preference. In contrast, consistent findings in the CeA of both P rats and HAD rats suggest that NPY in the CeA of P and HAD rats may contribute to the regulation of alcohol consumption. This is substantiated by a recent report showing that NPY-knockout mice drink significantly more ethanol, and transgenic mice that overexpress the NPY gene drink less alcohol, than wild-type mice. Together, the findings support the notion that NPY agonists that would enhance NPY function in the amygdala might be useful for the treatment of anxiety and alcoholism.

Alcohol Clin Exp Res 1998 Nov;22(8):1778-82

Neuropeptide Y levels in ethanol-naive alcohol-preferring and nonpreferring rats and in Wistar rats after ethanol exposure.

Ehlers CL, Li TK, Lumeng L, Hwang BH, Somes C, Jimenez P, Mathe AA.

Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037, USA.

Neuropeptide Y (NPY) is a hexatriacontapeptide amide that is now well characterized as a neuromodulator in the central nervous system (CNS). When infused into the CNS, NPY produces both anxiolytic and orexigenic effects. NPY's anxiolytic effects appear to be mediated through receptors in the central amygdala, whereas its orexigenic effects are localized in discrete hypothalamic nuclei. Both food restriction and food deprivation produce increased levels of the peptide in the hypothalamus that are ameliorated by refeeding. However, the effects of alcohol consumption/deprivation on NPY levels remain unknown. The present study sought to determine if brain NPY levels were affected by either alcohol exposure and/or correlated with genetic differences in preference for drinking alcohol. In the first experiment, NPY-like immunoreactivity (NPY-LI) was compared in alcohol-naive, alcohol-preferring (P), and nonpreferring (NP) rats. After tissue extraction, NPY-LI was measured by radioimmunoassay: amygdala, hippocampus, frontal cortex, hypothalamus, and caudate. P rats were found to have significantly lower NPY-LI in amygdala ($F = 4.69$, $p < 0.04$), hippocampus ($F = 7.03$, $p < 0.01$); and frontal cortex ($F = 4.7$, $p < 0.04$), compared with NP rats. In the second experiment, heterozygous Wistar rats were exposed to alcohol for 14 hr/day for 7 weeks in alcohol vapor chambers (mean blood alcohol concentrations = 180 mg%) or control chambers. At 7 weeks of alcohol exposure, no significant changes in NPY-LI in were found. At 1 month after ethanol withdrawal, however, the ethanol-exposed animals had significantly higher NPY-LI in the hypothalamus ($F = 4.78$, $p < 0.04$) when compared with the nonexposed controls. Taken together, these studies suggest that exposure to chronic ethanol may affect NPY-LI at the level of the hypothalamus in a fashion similar to food restriction, because 4 weeks after alcohol withdrawal, significantly higher NPY levels are found. In addition, differences in NPY-LI in limbic areas and frontal cortex between alcohol-naive P and NP rats suggest that NPY may also play a role in risk for the development of alcohol preference either by modulating the "tension-reduction" properties of alcohol or by influencing consummatory behaviors.